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Detection of Mutations in the Enhancer 2/Core Promoter Region of Hepatitis B Virus in Patients With Chronic Hepatitis B Virus Infection: Comparison With Mutations in Precore and Core Regions in Relation to Clinical Status

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To investigate the meaning of the mutations in the enhancer 2/core promoter (Enh2/CP) region of hepatitis B virus (HBV) during the chronic HBV infection, mutations were examined in the Enh2/CP region (carboxyl half of X region) and their correlation with mutations in the precore and core regions in relation to the presence of chronic liver disease. The entire nucleotide sequences of the Enh2/CP region were determined by direct sequencing of the amplified products derived from 30 cases with chronic HBV infection. The results were compared to the mutations in the precore and core regions. In the Enh2/CP region, 91 generally scattered nucleotide substitutions were detected. There were 11 substitutions in the 10 asymptomatic healthy carriers (mean, 1.1/case) and 80 in the 20 chronic liver disease patients (4.0/case). The most frequent substitutions from A to T at nucleotide 1764 and from G to A at nucleotide 1766 were seen in none of the 10 asymptomatic carriers and in 14 (70%) of the 20 chronic liver disease patients. Comparisons of mutations in the precore and core regions revealed that 14 of 16 patients with mutations in the core region had the mutations in the Enh2/CP region and/or a precore stop codon mutation. These data suggest that mutations in the Enh2/CP and precore regions may affect the expression of the core and HBeAg peptides and might be involved in the pathogenesis of chronic liver disease. ***J. Med. Virol. 57:337–344, 1999.***

KEY WORDS: asymptomatic healthy carriers; chronic liver disease; X region; precore region; core region

INTRODUCTION

Individuals infected with hepatitis B virus (HBV) can be divided clinically into those who are asymptomatic healthy carriers and those with chronic liver disease. A previous report suggests that HBV with mutations in the nucleocapsid core region is closely associated with the development of chronic liver injury in asymptomatic carriers [Ehata et al., 1992]. Recent studies have revealed that point mutations in the basic core promoter (BCP) region, located in the carboxyl half of the Enh2/CP region, are also common in patients with chronic liver disease [Okamoto et al., 1994; Laskus et al., 1995; Kurosaki et al., 1996]. The mutation in the precore promoter is considered to occur during seroconversion from hepatitis B e antigen (HBeAg) to antibody (anti-HBe) [Okamoto et al., 1994]. The enhancer 2/core promoter (Enh2/CP) region, sharing its sequence with the X region, was reported to play a role in the regulation of expression of hepatitis B core antigen (HBcAg) and HBeAg peptides [Yuh et al., 1992]. Investigation of mutational changes in the regions responsible for production of HBcAg and HBeAg peptides may provide a clue for revealing the mechanism(s) of chronic liver injury.

We analyzed the mutations in the Enh2/CP region in the same sera from 30 HBsAg-positive cases in which we had examined the mutations in the precore and core regions: 10 asymptomatic healthy carriers and 20 patients suffering from chronic liver disease with fluctuating serum alanine aminotransferase (ALT) levels [Ehata et al., 1992]. The nucleotide sequence of the carboxyl half of the X region, including the entire BCP

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TABLE I. Clinical and Laboratory Data of 10 Asymptomatic Carriers and 20 Patients With Chronic Liver Diseases

Case	Age/sex	Liver histology	ALT (IU/L)	Follow-up (year)	HBeAg/ HBeAb	Enh2/CP mutations (nucleotides 1764, 1766)	Precore mutation (codon 28)	Core mutation
Asymptomatic carriers								
1	27/F	ND	17	8.0	+/–	–	–	–
2	39/F	NSC	17	8.0	+/–	–	–	–
3	48/M	ND	22	4.2	+/–	–	–	–
4	20/M	ND	34	7.0	+/–	–	–	–
5	24/M	ND	29	4.0	+/–	–	–	–
6	26/M	NSC	31	5.0	+/–	–	–	–
7	21/F	ND	19	7.0	+/–	–	–	–
8	41/M	CPH	22	4.5	+/–	–	–	–
9	30/F	NSC	34	3.0	+/–	–	–	–
10	17/F	CPH	18	7.0	+/–	–	–	–
Patients with chronic liver diseases								
11	43/M	CPH	278	9.0	+/–	+	–	–
12	49/F	CPH	126	4.0	+/–	+	–	–
13	30/M	CPH	107	3.0	+/–	+	–	–
14	52/F	CPH	337	9.0	+/–	+	–	+
15	40/F	ND	131	3.0	+/–	–	–	–
16	38/M	CAH	395	8.0	+/–	+	–	+
17	38/M	LC	86	8.0	+/–	–	+	+
18	35/M	CAH	110	10.0	+/–	+	–	+
19	42/M	CAH	114	10.0	+/–	+	–	+
20	29/F	CAH	537	8.0	+/–	+	–	+
21	41/M	CAH	131	9.0	+/–	–	–	+
22	31/M	CAH	846	8.0	+/–	+	–	+
23	24/M	CAH	849	4.0	+/–	+	–	+
24	22/M	CAH	833	6.0	+/–	+	–	+
25	32/F	CAH	33	8.0	+/–	+	+	+
26	46/M	CAH	251	11.0	–/+	+	+	+
27	42/M	CAH	503	3.0	–/+	–	–	+
28	26/M	CAH	588	9.0	–/+	+	–	+
29	30/M	CAH	598	6.0	–/+	–	+	+
30	34/F	CAH	537	6.0	–/+	–	+	+

region, was determined in these 30 cases by polymerase chain reaction (PCR) and direct sequencing method. The association of mutations in these regions and mutations in the precore and core regions was studied in relation to the presence or absence of liver disease.

MATERIALS AND METHODS

Patients

Sera were obtained from 30 persistent carriers of hepatitis B surface antigen (HBsAg), who were followed at the First Department of Medicine, Chiba University, for at least 3 years. These cases all had the *adr* subtype of HBsAg. They were examined for mutations in the core and precore regions of HBV, and detailed results were already described [Ehata et al., 1992]. They consisted of 10 HBeAg-positive asymptomatic healthy carriers and 20 patients with chronic liver disease.

The asymptomatic subjects generally showed serum ALT levels to be within the normal range by regular examinations every 1–2 months for 3–8 years. Liver biopsy was performed in five, and only mild histological changes (chronic persistent hepatitis in two and nonspecific changes in three) were found (cases 1–10, Table I).

Of the 20 patients with fluctuating liver enzymes, 15 (cases 11–25, Table I) had HBeAg-positive chronic liver disease. Liver biopsy in 14 of these patients revealed chronic persistent hepatitis in 4, chronic active hepatitis in 9, and cirrhosis in 1. The other five patients (cases 26–30, Table I) had anti-HBe–positive chronic liver disease, and liver biopsy led to their histological diagnosis of chronic active hepatitis. There was no statistical differences in age and duration of follow-up between asymptomatic carriers and patients with chronic hepatitis. Informed consent was obtained from each patient. This study was approved by the ethics committee of Chiba University School of Medicine.

HBV Markers

HBsAg, HBeAg, and anti-HBe were detected by solid-phase radioimmunoassay (Abbott Laboratories, North Chicago, IL). Subtypes of HBsAg were assayed by enzyme immunoassay (Special Immunology Laboratories, Tokyo, Japan). Second-generation anti-hepatitis C virus antibody was measured by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan), and all samples were negative.

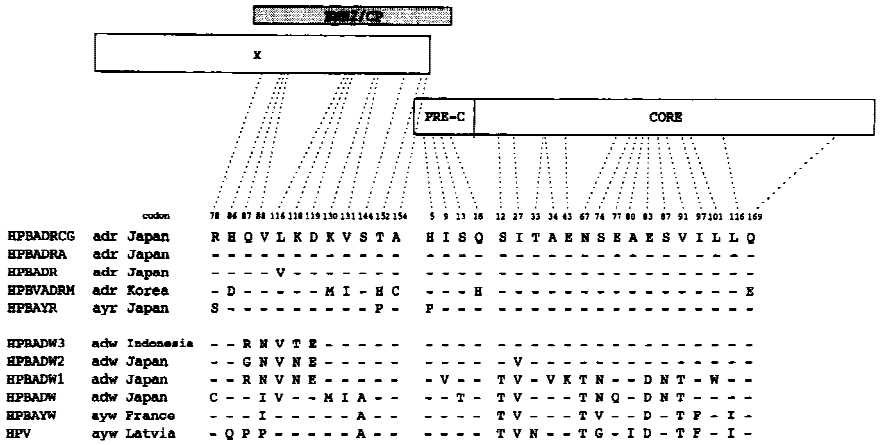

Fig. 1. Comparison of deduced amino acid sequences of carboxyl half of X and precore and core regions. Amino acids different from HPBADRCG are plotted against the corresponding positions of amino acids. The dashed lines indicate the same amino acid as HPBADRCG. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Quoted references are as follows: HPBADRCG [Fujiyama et al., 1983]; HPBADRA [Kobayashi and Koike, 1984]; HPBADR [Ono et al., 1983]; HPBVADRM [Roh et al., 1989]; HPBAYR [Okamoto et al., 1986]; HPBADW3 [Okamoto et al., 1988]; HPBADW2 [Okamoto et al., 1988]; HPBADW1 [Okamoto et al., 1988]; HPBADW [Ono et al., 1983]; HPBAYW [Galibert et al., 1979]; and HPV [Bichko et al., 1985].

Amplification and Sequencing of Enh2/CP Region of HBV DNA

To amplify the Enh2/CP region, which overlaps the carboxyl half of the X region, several sets of synthetic oligonucleotide primers were prepared based on the HBV DNA sequence (HPBADRCG) reported by Fujiyama et al. [1983]. The sequences of these primers were: sense primer, X2 (nucleotides 1553–1573, 5′-GTCTGTGCCTTCTCATCTGCC-3′); antisense primers, C2B (nucleotides 2062–2081, 5′-AGAATAGCTTGCCTGAGTGC-3′) and C13B (nucleotides 1982–2001, 5′-GGCAAAAAAGAGAGTAACTC-3′). With these primers, a segment of HBV DNA spanning from nucleotides 1553–2001 comprising the Enh2/CP region was amplified. Amplification of HBV DNA was basically performed as described previously [Yokosuka et al., 1991]. Briefly, 100 μl of reaction mixture containing 10 μl of specimen DNA extracted from 50 μl of serum, 50-mM KCl, 10-mM Tris-HCl (pH 8.4), 2.5-mM $MgCl_2$, 1 μM each of the two oligonucleotide primers X2 and C2B, 200-μM dNTP, 200 μg/ml of gelatin, and 2 units of Thermus aquaticus DNA polymerase was overlaid with 100 μl of mineral oil. Samples were heated at 94°C for 1 min, cooled to 55°C for 1 min, and then heated to 72°C for 1 min. These steps were repeated for 40 cycles. After the final step of amplification, 2 μl of the first amplification product was mixed with the same reaction mixture except for the antisense primer C13B instead of C2B. Second-step amplification was performed under the same conditions, and each sample (10 μl) was applied to an 8% acrylamide gel along with molecular size markers. The amplified segments were centrifuged in microconcentrators (Centricon 30; Amicon, Danvers, MA) for direct sequencing.

Sequence primers were prepared for bidirectional sequencing: sense primer, X7 (nucleotides 1646–1665, 5′-AAGGTCTTACATAAGAGGAC-3′) and antisense primer, X2B (nucleotides 1775–1794, 5′-TTATGCCTACAGCCTCCTAG-3′). Direct sequencing was performed as previously described [Tada et al., 1990]. Briefly, one sequence primer was radiolabeled with ^{32}P-ATP and T4 polynucleotide kinase. Approximately 10 pmol of microconcentrator purified PCR product and 5 pmol of ^{32}P-labeled sequencing primer were combined in 12 μl of 50-mM KCl, 50-mM Tris-HCl (pH 8.0), 5-mM $MgCl_2$, and 10-mM dithiothreitol, heated at 80°C for 5 min, and then immediately chilled on ice. Three μl of the mixture was added to dideoxy reaction mixture and reacted with Sequenase (Sequenase version 2.0, USB, Cleveland, OH) according to the instructions of the manufacturer. The reaction mixture was electrophoresed on a 40-cm sequencing gel. After drying the gel, autoradiography was performed for 24–72 hr.

The sequences of precore (87 nucleotides/29 amino acid residues) and core (549–555 nucleotides/183–185 amino acid residues) regions in these 30 patients were examined by the same direct sequence method as previously described [Ehata et al., 1992].

Statistical Analysis

Values were expressed as mean ± standard deviation and statistical analysis was done by Student's *t*-test, and P values of <0.05 were considered significant.

RESULTS

Analysis of Amino Acid Sequences

To determine whether the amino acid alterations were the result of deviations of subtypes or were true mutations, 11 previously reported strains were compared in terms of their amino acid sequences of the carboxyl half of the X, precore, and core regions (Fig. 1). Five sequences with *adr* or *ayr* subtypes showed well-

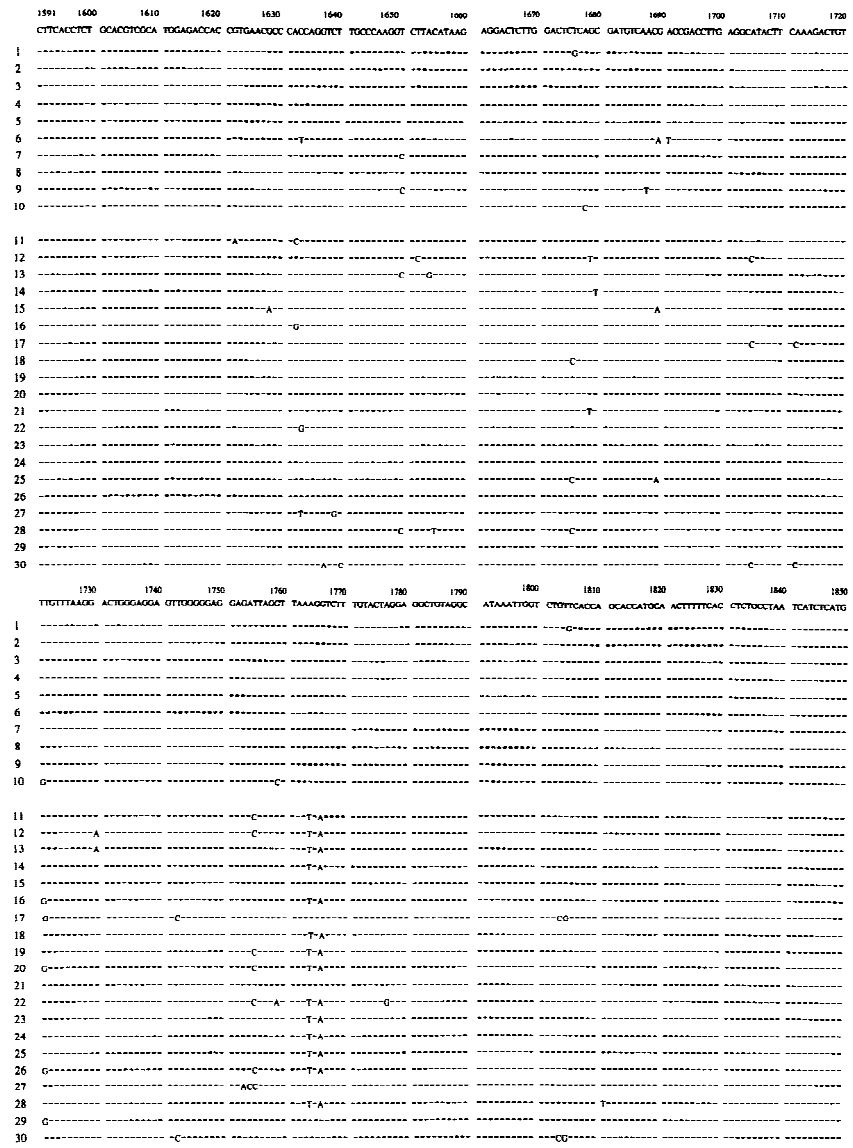

Fig. 2. Nucleotides sequences of Enh2/CP region in 30 cases. Nucleotide numbers and HPBADRCG sequence are given in the top two rows [Fujiyama et al., 1983]. Dashes indicate homology with HPBADRCG. Nucleotides different from HPBADRCG are shown.

conserved amino acid residues of the carboxyl half of the X region and precore and core regions. In contrast, HBV with the "*w*" determinant had slightly different sequences. The codons showing amino acid residues different from the prototype *adr* sequence against corresponding positions of the carboxyl half of the X, precore, and core regions were plotted. Since all the patients examined had the *adr* subtype, the HPBADRCG sequence was selected as the representative for further analysis.

Nucleotide Sequences of Enh2/CP Region and Deduced Amino Acid Residues of Carboxyl Half of X Region

The entire nucleotide sequences of the Enh2/CP region (nucleotide 1591–1840) were analyzed in all 30 cases and were compared with the representative HPBADRCG sequence (Fig. 2). The amino acid residues deduced from the nucleotide sequences of the carboxyl half of the X region (amino acid 73–154) were also compared with the deduced amino acid of HPBADRCG (Fig. 3).

There were nucleotide substitutions at 91 locations, of which 70 were "missense" (alteration in the deduced amino acid residue) and 21 "silent" (no alteration) (Fig. 2). There were 11 nucleotide substitutions in the 10 asymptomatic healthy carriers (mean, 1.1 ± 1.2) and 80 in the 20 chronic liver disease patients (mean, 4.0 ± 1.8). Deduced amino acid substitutions were 8 in the 10 asymptomatic carriers (mean, 0.8 ± 0.9) and 62 in the 20 patients (mean, 3.1 ± 1.5) (Fig. 3).

In the Enh2/CP region of HBV in the chronic liver disease patients, nucleotide and deduced amino acid substitutions were generally scattered, but three spe-

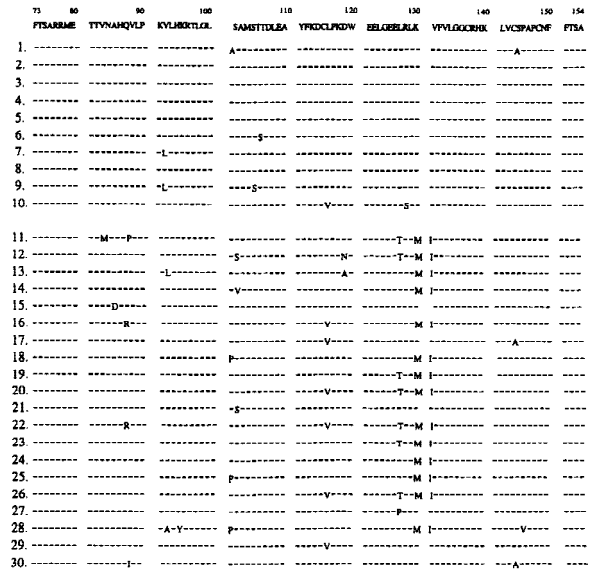

Fig. 3. Deduced amino acid residues of carboxyl half of X region in 30 cases. All amino acid residues different from HPBADRCG are shown. X codon numbers and HPBADRCG sequence are given in the top two rows. Dashes indicate homology with HPBADRCG. Amino acid residues are expressed by single letters (see legend for Fig. 1).

cific nucleotide and deduced amino acid substitutions were observed (Figs. 2 and 3). A at nucleotide 1764 and G at nucleotide 1766 changed to T and A, respectively, in 14 (70%) (12 HBeAg-positive and 2 anti-HBe–positive) of the 20 patients with chronic liver disease (Table I). In these 14 patients, corresponding to the substitutions of these nucleotides, lysine at X codon 130 was changed to methionine and valine at X codon 131 to isoleucine (Fig. 3). Also, the proline in codon 127 changed to threonine corresponding to the substitution of T at nucleotide 1755 to C in 8 of the 20 (40%) patients (Figs. 2 and 3). These substitutions were not found in the 10 asymptomatic healthy carriers (Fig. 3).

Of the 25 seropositive cases for HBeAg, the mean HBeAg cutoff values with and without the mutations at 1764 and 1766 in the core promoter region were 3.28 ± 0.66 (n = 12) and 4.05 ± 0.78 (n = 13), respectively, and tended to be lower in cases with the mutations compared to those without them, although the difference was not statistically significant.

Comparison of Mutations

The mutations in the Enh2/CP region examined in this study were compared to the amino acid sequences in the precore and core regions in the 30 patients, which were previously described [Ehata et al., 1992]. To sum up the previous data on the precore and core regions, the amino acid mutations in the core region were found in 16 (80%) of the 20 chronic liver disease patients (Fig. 4), including 12 patients (60%) (8 HBeAg-positive and 4 anti-HBe–positive) with mutations in the mutation clustering region in the core region (core codons 87–97) [Ehata et al., 1992]. No mutation was found in the remaining 4 patients (20%), nor in the 10 asymptomatic healthy carriers (Table I). Five (25%) of the 20 patients with chronic liver disease (2 HBeAg-positive and 3 anti-HBe–positive) had the stop codon mutation at precore codon 28, which inhibits the production of the HBeAg precursor (Table I) [Carman et al., 1989; Okamoto et al., 1990].

Together with the data in the current study, the specific mutations at nucleotide 1764 and nucleotide 1766 in the Enh2/CP region, the precore stop codon mutation at precore codon 28, and the mutation at the core region were not observed in the 10 asymptomatic carriers (Table I, Fig. 4). In contrast, 19 of 20 patients with chronic hepatitis B had one or more of the specific mutations at nucleotide 1764 and nucleotide 1766 in the Enh2/CP region, the precore stop codon mutation, and mutation at the core region (Table I, Fig. 4). In detail, 9 of the 16 patients with mutations at the core region had the mutations at nucleotide 1764 and nucleotide 1766 in the Enh2/CP region only, 3 had stop codon mutation at precore codon 28 only, and 2 had mutations both in the Enh2/CP region and in the precore stop codon mutation, meaning that a total of 14 of the 16 patients had either the specific mutations at the Enh2/CP region or stop codon mutation at precore codon 28. Three of the four patients without mutation at the core region had the specific mutations at the Enh2/CP region.

DISCUSSION

Hepatitis B virus infection leads to various types of liver disease. Although the mechanisms involved in the development of chronic liver diseases from HBV infection are not fully clarified yet, it is believed that immunological mechanisms are involved in the process [Van Hecke et al., 1994]. It was shown that the core antigen peptide and HBeAg peptide, which has an overlapping sequence with the core peptide [Takahashi et al., 1983; Uy et al., 1986; Standring et al., 1988], were recognized by cytotoxic T-lymphocytes (CTL) of the host [Mondelli et al., 1982; Vento et al., 1985; Ferrari et al., 1987; Milich et al., 1989]. A previous study revealed the presence of mutation clustering regions in the core region of HBV taken from patients with chronic liver disease but not from asymptomatic carriers [Ehata et al., 1992]. Therefore, mutational changes of core peptides might be associated with the development of chronic liver disease as a result of immunological attack.

The transcriptions of the precore and pregenome mRNA, messages for HBeAg and core protein, respectively, were reported to be regulated by the Enh2/CP region located just upstream of the precore region [Yuh et al., 1992]. In the Enh2/CP region there are three AT-rich regions: ATTA (nucleotides 1754–1757), TTAAA (nucleotides 1760–1764), and ATAAATT (nucleotides 1791–1797). The second AT-rich region in the Enh2/CP region has been proposed as the transcription site of precore mRNA, and the third AT-rich region as having a dual function, initiator for the tran-

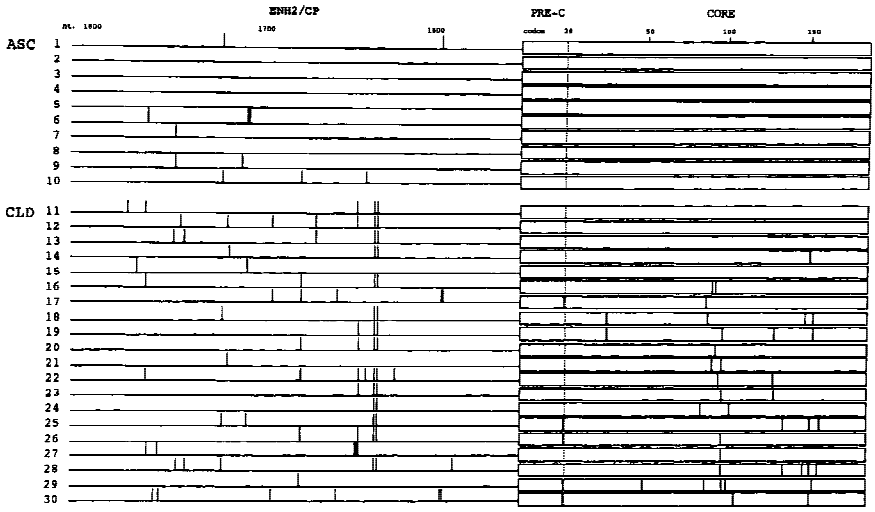

Fig. 4. Nucleotide mutations in Enh2/CP region and amino acid mutations in precore and core regions are shown. The vertical solid lines indicate the positions of the mutations. ASC, asymptomatic healthy carriers; CLD, patients with chronic liver disease.

scription of precore mRNA and a TATA element for pregenome mRNA [Yaginuma et al., 1987; Chen et al., 1995].

In the current study, a number of substitutions were found within the Enh2/CP region, and those at nucleotides 1753–1766, nucleotides 1676–1680, and nucleotides 1632–1640 were particularly common (Fig. 2). The changes from A to T at nucleotides 1764 and from G to A at nucleotides 1766, which affect the second AT-rich region, were observed most frequently, as already described by others [Okamoto et al., 1994; Laskus et al., 1995; Kurosaki et al., 1996]. They were detected specifically in 14 (70%) (12 HBeAg-positive and 2 anti-HBe–positive) of the 20 patients with chronic liver disease (Fig. 2). These changes were not found in the 10 asymptomatic healthy carriers. Taking into account the fact that point mutations in the TATA sequence motif in eukaryotic promoters drastically change specific in vitro transcription [Wasylyk et al., 1981; Myers et al., 1986], it is possible that these mutations are closely associated with the transcription of precore mRNA. Actually, the HBeAg titer was lower in the cases with the mutation than in those without, although this was not statistically significant. It was recently reported that these mutations are accompanied by a reduced transcription of precore mRNA and a lower expression of HBeAg peptide [Moriyama et al., 1996]. However, the third AT-rich region (nucleotides 1791–1797) was completely preserved in our patients (Fig. 2), compatible with the reports by Okamoto et al. [1994] and Laskus et al. [1995], suggesting that the role of this AT-rich region may be indispensable for the survival of the virus. The results of Kurosaki et al. [1996] were in disagreement, the reasons for which are not yet clear.

The expression of precore mRNA might also be regulated by transactivator X protein affecting host cell genes such as RNA polymerase II [Aufiero and Schneider, 1990]. We also focused on the deduced amino acid residues of the carboxyl half of the X region. Previous studies have reported the two domains responsible for the transcriptional function of the X region [Ritter et al., 1991; Arii et al., 1992]. One is a binding domain around X codons 32–66 in the amino half of the region, and the other an activating site enriched with acidic amino acids around X codons 106–148 in the carboxyl half. In our study, corresponding to the nucleotide mutations in the Enh2/CP region, lysine at X codon 130 altering to methionine, valine at codon 131 to isoleucine, and proline at codon 127 to threonine were distinguishable only in chronic liver disease patients (Fig. 3). These mutations could have some effect on the transactivating function of X protein. In other words, the mutations in the Enh2/CP region may also affect the overlapping X open reading frame and its function as transactivator in the mechanisms of viral replication.

In this study, examination of the incidence of mutations in the Enh2/CP and/or precore and core regions of the HBV genome (Table I) revealed that most of the patients with chronic liver disease had mutations in either Enh2/CP and/or precore and/or core regions, providing evidence that they are associated with the clinical state of liver disease. Mutations in the core region were found in 16 of 20 chronic liver disease patients, especially in the more severe forms of liver disease, and they were considered to be immunological target sites of CTL [Ehata et al., 1992].

The nucleotide mutations of nucleotide 1764 and nucleotide 1766 in the Enh2/CP region and/or the precore stop mutation at codon 28 were recognized in 17 (85%) of 20 chronic liver disease patients (Table I). The nucleotide mutations in the Enh2/CP region could reduce the transcription of precore mRNA and the expression of HBeAg peptide [Moriyama et al., 1996], and the precore stop mutation might then inhibit the production of HBeAg due to the incapability of translation of the precore message [Carman et al., 1989; Okamoto

et al., 1990]. Thus, these changes could either reduce or stop the production of HBeAg peptide. It may be that these mutations occurred as a result of the pressure from host immunological responses to the virus because the reduction or cessation of the production of HBeAg, also a possible immunological target of CTL, could escape from the attack from CTL, a situation favorable for the survival of the virus.

Howerer, this raised the contradiction that three chronic liver disease patients without mutations in the core region had mutations in the Enh2/CP region (Table I, cases 11–13). All three cases had only mild hepatitis, and therefore there is a possibility that the reduction of HBeAg peptide caused by mutation in Enh2/CP might trigger the recognition of core and HBeAg peptides by immunological cells, because the HBeAg peptide was suggested to be a tolerogen from immunological attacks [Milich et al., 1990]. However, since the amplified product was sequenced directly and the changes of the dominant-type virus were noted, the possibility of the existence of a minor population having mutation(s) in the core region could not be totally excluded. The follow-up of our asymptomatic patients without specific mutations for the possible emergence of such mutations might reveal which of the mutations emerge first, and would also be helpful for evaluating the predictability of the development of liver disease.

Chen et al. [1992] have reported that the precore gene of woodchuck hepatitis virus (WHV) is not essential for viral replication by applying mutant WHV with precore stop codon to woodchucks. They also demonstrated the importance of WHV X gene in initiating or maintaining replication in vivo by introducing stop codon mutations in the X gene of WHV with in vitro mutagenesis [Chen et al., 1993]. To further understand the meaning of the specific mutations in the Enh2/CP region in the development of liver injury, in vivo studies will have to be performed using animal models such as the woodchuck and by applying viruses with the corresponding specific mutations in the Enh2/CP region.

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